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Qualifying Fat Storage Phenotypes Using Gas Chromatography in Caenorhabditis elegans

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Abstract

With the emerging role of *C. elegans* as a model for the basic biology of metabolic disorders, quantitating fat in worms has become increasingly important. Current methods involve staining worms for fat deposits to study changes in metabolic pathways; however these methods can be subjective and produce misleading information. In this study, gas chromatography was used to analyze lipid content in worms and investigate if there is a ratio of fatty acids that can distinguish fat increased from fat decreased mutants. A method was established to prepare lipid samples utilizing alkaline esterification of lipids to produce fatty acid methyl esters. Then this method was used on mutants with reported fat phenotypes. After analyzing their lipid profiles, I found no such fatty acid ratio exists. A strong degree of fatty acid homeostasis was observed regardless of different variables which can be investigated in future studies.

Introduction

Caenorhabditis elegans has become an important model organism for metabolic defects because the basic biology of metabolic processes is conserved in worms (Jones & Ashrafi 2009). Like mammals, *C. elegans* utilize a range of mono and polyunsaturated fats as well as monomethyl branched chain fatty acids (mmBCFAs) (Ashrafi 2007; Kniazeva 2004). There are several key differences between mammals and *C. elegans*. For instance, *C. elegans* store fat in intestinal and epidermal cells. Worms are capable of synthesizing the essential fatty acids linoleic acid (18:2n6) and linolenic acid (18:3n3); worms do not require leptin to store fat, and do not exhibit the same health issues of excess fat accumulation (Mullaney and Ashrafi 2009; Ashrafi 2007). Despite the differences in fat metabolism between mammals and worms, *C. elegans* genetics can be easily manipulated using both forward and reverse genetic techniques which is useful in the laboratory setting. In addition to conserved processes, over 70% of the

C.elegans lipid genes have a human ortholog (Zhang et. al 2013). By genetic manipulation, it is possible to understand the genetic regulation and basic biology behind obesity which may offer insight on how to treat it (Freidman et. al 2003).

Studying fat storage defects requires being able to detect relative changes in fat storage in worms. Current methods of visualizing fat take advantage of the translucency of the nematode by using fluorescent lipophilic stains. The main advantage of staining is it does not disrupt *C. elegans* development. Commonly used stains include Sudan Black and Oil Red—both of which require fixation. Staining with Nile Red or feeding worms BIODIPY-labeled fatty acids can be used to stain live worms offering a dynamic image of fat in the worm. However there are limitations to staining. Sudan Black staining can be hard to quantify. Nile Red has been shown to poorly stain tissues known to be high in fat like the germline and hypodermis, producing false negative phenotypes. Both Nile Red and BIODIPY-labeled fatty acids stain for lysosome-related organelles which are not fat storage sites in the worm, producing false positives. Improvements in staining have shown Nile Red stains fixed worms better than live ones, and Oil Red is thought to provide the best picture of fat in worms because the intensity of the stain decreases as worms are starved longer (Watts 2009; O'Rourke et al 2009).

When a fat storage phenotype is reported in the literature, multiple pieces of evidence are shown. Evidence can consist of using to different stains, pumping rate, food absorption rate, staining another organism, and comparison of fat content to protein content (Wang et. al 2008; Van Gilst et. al 2005; Ashrafi et. al 2003; Lemieux et. al 2011). By these methods, fat phenotypes are reported when multiple pieces of evidence point to the same conclusion. Recently gas chromatography (GC) has been used to characterize *C. elegans* with irregularities in fatty acid composition. For example, loss of function mutants in the fatty acid elongase gene, *elo-5*,

show an altered fatty acid composition by GC. These mutants lack the ability of making monomethyl branch chain fatty acids. The lack of mmBCFAs causes worms to arrest at the first larval state (L1). Using GC, Kniazeva et. al showed *elo-5(lf)* have no mmBCFAs causing this L1 arrest (2004). Because GC analysis can reveal what fatty acids are present, GC may also be advantageous in quantitating fat in worms due to its speed, efficiency, and objectivity. Perhaps by using GC it is possible to observe a ratio of areas of certain fatty acids that can determine if a worm has increased or decreased fat content. If such a ratio exists, this would be a more objective approach to characterize if a worm has increased or decreased fat content compared to wild type.

This study establishes a new methodology in determining if a worm has altered fat content that relies on lipid profiles generated with GC. First, a basic lipid esterification method was designed and optimized to prepare GC analysis. Using GC, I show a detectable change in total fat in using different fed states giving me confidence that changes in fat content may be detectable as well. In addition, I show that the lipid composition does not fluctuate drastically at various culturing temperatures. Finally, mutants reported with increased fat only show a minor enrichment in fatty acids in triacylglycerol (TAG) using GC analysis, but within error the increase is not significant.

Methods and Materials

Acid and Base Sample Preparation

Synchronized populations of well-fed N2 were collected with water off of Nematode Growth Media (NGM) petri dishes seeded with OP50 *E. coli*. Samples were then rinsed 2-3 times then the excess water was aspirated off. For basic preparation, samples were placed in Eppendorf tubes (catalog no. 22363212) before 1ml of hexane was added to the samples. Then

100µl of Methanol+2M KOH solution and was vortexed for two minutes (except for time dependent incubation experiments where reaction time is varied from 2 to 160 minutes). For acid preparation, samples were transferred to glass tubes and aspirated of excess water then were incubated with 1ml of Methanol+2.5% H_2SO_4 for one hour at 80^{o}C. Then 1ml of hexane was added in two 500µl additions once the samples cooled. Samples prepared using either method were spun down at 1400rpm for one minute and the upper organic layer was extracted for GC analysis. Samples were then analyzed on the HP6890N (Agilent, Palo Alto, California, United States) equipped with a DB-23 column (30 m × 250 µm × 0.25 µm) using the same settings described by Kniazeva et al. (2003).

Testing Fed States

N2 embryos were spotted, then the worms were allowed to grow up to L4 larval stage. At L4, worms were collected off the NGM/OP50 plates, washed once with M9, and then re-plated back onto NGM/OP50 or empty NGM plates either overnight or two days. Populations placed on empty NGM were considered starved populations. Samples were collected in triplicate, prepared using basic method, and analyzed as previously described. Experiments were repeated twice with averages and standard deviations reported.

Generational Temperature Dependence

N2's were bleached-synced and grown at 15^{o}C, 20^{o}C, and 25^{o}C. GC samples were collected when worms were gravid adults. When adults were washed away, the NGM/OP50 plates were kept in order to allow the F1 generation to grow to gravid adults. Then the F1's were bleached and used to seed the second generation (F3). This process was carried out for up to three generations being sure to keep worms at assigned temperature (15^{o}C, 20^{o}C, or 25^{o}C) when samples were not being collected or prepared.

Testing Fat Mutants

Strains that have been reported to have increased fat content were obtained—*age-1(hx546), aak-2(gt33), nhr-49(2041),* and *tph-1 (GR1321/MT1543).* Reported decreased fat *glp-1(ar202)* and increased fat mutant *glp-1(e2141ts)* were also obtained (Lemieux et. al 2011; Ogg & Ruvkun, 1998; Van Gilst et. al 2005; Ashrafi et. al 2003; Wang et. al 2008). Strains were kept well fed for two generations with bleaching in-between generations before beginning the experiment. Samples were collected in triplicate in four independent experiments performed every two weeks. Averages per each experiment were calculated independently. The average and standard deviations of the four experiments are reported.

Oil Red Staining

Oil Red staining was performed as outlined in O'Rourke et. al (2009) except worms were incubated in oil red solution for five hours. Images of lower gonad regions were taken using Nomarski and GFP filter. Pictures were analyzed using ImageJ software to adjust he threshold of the image so major lipid stores are visible then mean image density was calculated.

GC profile analysis

Lipid profiles were analyzed using GC Chem Station software that is compatible with the GC. Integration settings first established a height and area reject limit to eliminate the background noise. These settings where then applied to all lipid profiles generated that day. Then area values were matched based on their retention times and labeled with the corresponding fatty acid. Area percent was calculated from total area accordingly.

Results

Lipid Extraction using KOH and Methanol Yields More Polyunsaturated Fatty Acids and is Dependent on Reaction Duration.

Two methods of lipid extraction are currently being used in lab. One method, termed basic method, converts fatty acids into methyl esters (FAMEs) using a solution of methanol+2M KOH. Under normal laboratory conditions, this reaction occurs in two minutes which shortens the duration of procedure with high recovery. However the basic method can only detect esterified fatty acids thus not all fatty acids are analyzed. The second method, termed acidic method, requires the sample to be incubated in methanol+2.5% H_2SO_4 for one hour at 80^{o}C to generate FAMEs to be quantified using GC. This method is able to detect both esterified and free fatty acids, but has low yield and the procedure takes more time to do.

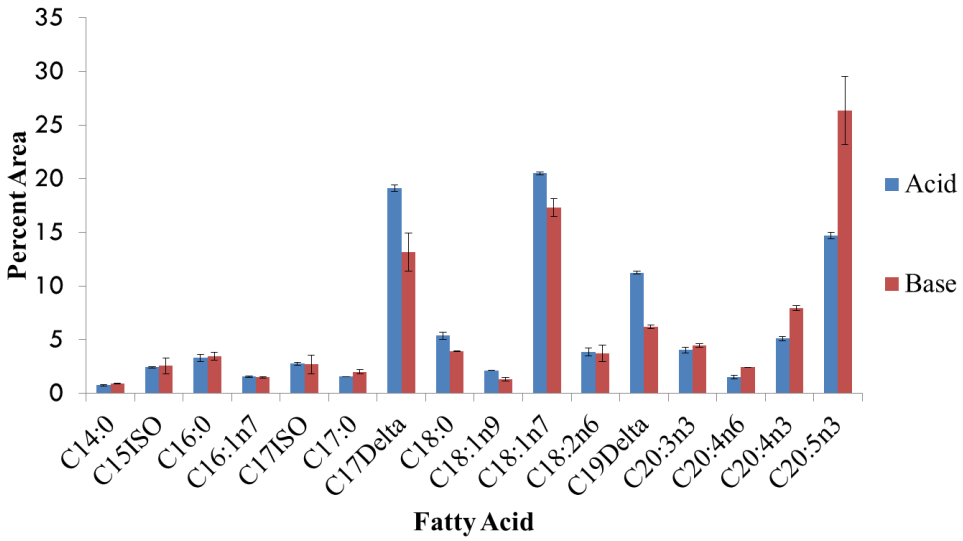

Figure 1- Wild type FAME samples were prepared using acidic or basic method. Samples were analyzed using GC. Each experiment was repeated twice in triplicate. Average lipid composition is shown along with standard deviation.

Since both methods have their advantages and disadvantages, it is necessary to compare the two methods using wild type worms in order to decide on a method to be used for the entire experiment. Figure 1 shows the relative amount of each fatty acid present from wild type (N2) whole worm lysate prepared using both the acidic and basic method. In general, the samples prepared using the acidic method contain more cyclopropane fatty acids because the main source of these are OP50 *E. Coli* thus these the additional free fatty acid comes from the bacteria in the intestine when worms were collected for analysis (Kaul et. al 2014). Conversely the basic method is

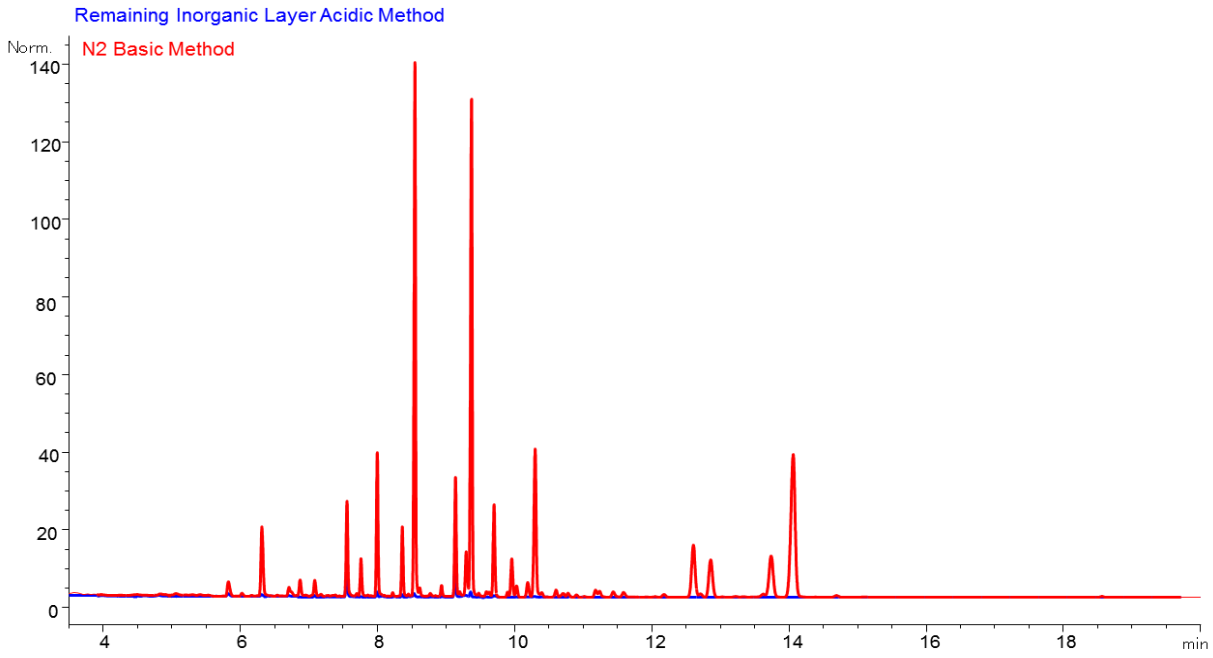

Figure 2-GC lipid profile of wild type (N2) FAME sample prepared using basic method. The remaining inorganic layer from the basic extraction was prepared for GC analysis using acidic method. The two GC readouts are overlaid and labeled accordingly. The inorganic layer essentially matches the baseline of the N2 profile indicating the basic method can extract most of the lipids in the sample.

relatively better at detecting polyunsaturated fatty acids (PUFAs) resulting in more PUFAs in lipid profiles. Since the main purpose of the experiments is to have a better indication fat phenotypes which would consist of more triacylglycerol esterified fatty acids, I opted in using the basic method to prepare FAME samples.

In order to test how well the basic method extracts all the esterified lipids, adult wild type worms were collected and prepared using the basic method. Afterwards the remaining inorganic layer was analyzed using the acidic method because both free and esterified fatty acids can be detected. Thus if there was anything lipid remaining in the inorganic layer, GC should be able to detect it. Since free fatty acids do not constitute a significant amount of total lipid, the signal of inorganic layer has the same intensity of the baseline indicating most of the fatty acid was extracted using the basic method (figure 2).

It was observed earlier that longer incubation time resulted in increased PUFAs (data not published). To further establish the method of preparing FAMEs for analysis, the incubation time called for in the basic method was varied in order to see what effects longer incubation time had on the lipid profiles.

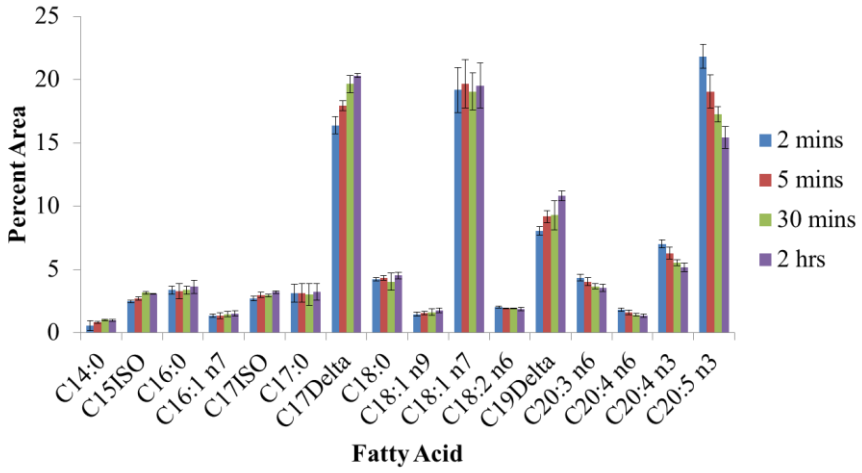

Figure 3-Reaction Duration of formation of FAMEs was varied from 2-120mins. . Each time was tested twice with samples ran in triplicate. Triplicates were averaged together to constitute that experiment. Figure 1 shows the average percent area of total lipid composition of two experiments with standard deviations from biological error.

Methanol/KOH incubation duration was varied at 2, 5, 30, and 120 minutes. These experiments were repeated twice and averages are reported in figure 3. In contrast to previous experiments, I found that the longer incubation time results in less PUFAs. Thus the incubation time was chosen to be 2 minutes long for future FAME preparation because this is believed to produce the truest lipid profile of the worms at the time of collection.

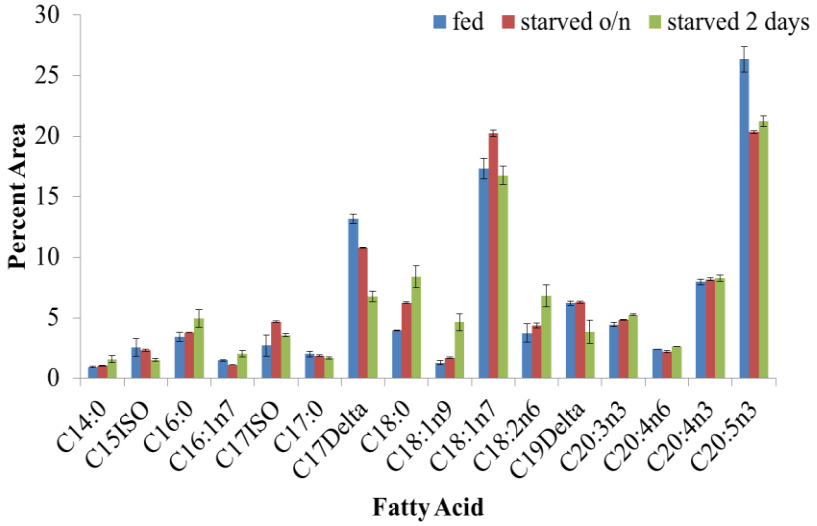

Figure 4- N2 worms were subjected to different fed states in order. Fed state is defined as worms given abundant food source while the starved states are is L4 worms being placed on empty NGM and kept on NGM either overnight (o/n) or for two days. Samples were prepared in triplicate and prepared using basic method. Experiment was repeated twice. Average lipid composition was calculated per experiment then the experiments were averaged together in figure 2. Standard deviation from biological error is reported.

Cyclopropane Fatty Acids in C. elegans can be detected using

GC and are indicative of Feeding Status.

Next I wanted to see that altered lipid profiles can be seen using GC. Wild type eggs were plated on lawns of OP50 and were allowed to grow to L4's. At that time, worms were washed twice with M9 and plated onto OP50 or empty NGM petri dishes. Half the worms plated on empty NGM and OP50 were collected the next day and the other half of the worms were kept on empty NGM for an additional night or two days before being collected for GC analysis using basic method. From figure 4, it can be seen that there are some changes in lipid content with varying degrees of starvation. The most significant change is in cis-9,10-methylenehexadecanoic acid (C17Δ) where the amount present decreases the longer the worm is starved. Cyclopropane fatty acids are synthesized by *E. coli* in the stationary phase. When worms consume *E. coli*, the fatty acid gets incorporated into triglyceride and phospholipids thus the longer worms are starved (lacking food), the less C17Δ present (figure 4). The amount of C17Δ will not diminish entirely because worms have a cyclopropane synthase capable of making C17Δ from monounsaturated fatty acids, but the level of C17Δ can be used as an indicator of well worms are fed (Kaul et. al 2014).

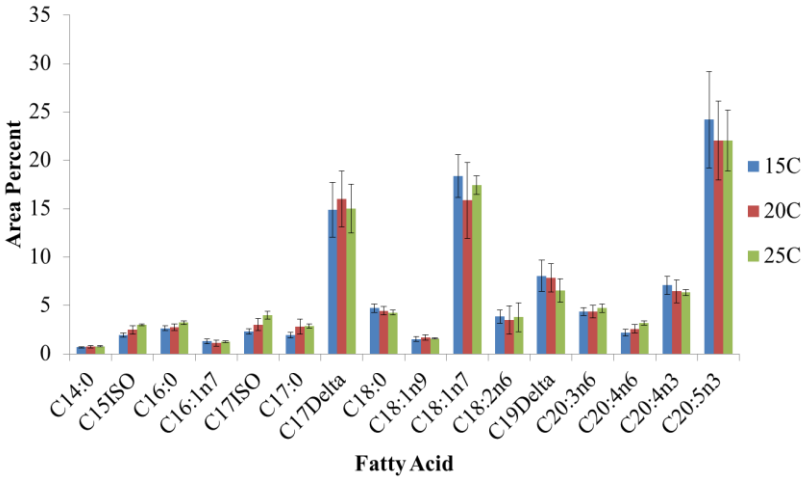

Figure 5-N2 worms were kept at various common incubation temperatures in order to see if temperature affects lipid profiles. Worms were followed out for five generations per tested temperature with bleach synchronization occurring in-between each generation. A generation consisted of three samples. Experiments were repeated three times. Data shown are average ± standard deviation

Temperature Does Not Alter Lipid Composition Significantly

Next, I wanted to see if temperature affected the fatty acid content in worms because some fat storage mutants grow better at

different temperatures. Therefore, I wanted to see if wild type worms grown at different temperatures produce comparable lipid profiles. Tested temperatures are common laboratory temperatures—15^{o}C, 20^{o}C, and 25^{o}C. Wild type worms were allowed to grow to gravid adults before samples were collected and prepared using basic method (figure 5). Eggs that were left on the plate once a sample was collected were allowed to grow to gravid adults which were then bleached and used to seed second generation. This procedure was carried out for 5 generations with 3 samples of each generation taken per temperature. Within error, the amount of fatty acid present does not change significantly with temperature thus fat storage mutants that are temperature sensitive can be compared to other mutants grown at a different temperature.

Oil Red Staining Suggests tph-1(GR1321/MT1543) has an Increased Fat Phenotype

Previously, *tph-1(mg280)* has been shown to have an increased fat phenotype (Ashrafi et. al 2003). However this strain has been reported to contain a background mutation that interferes with the phenotype. The new outcrossed strain *(GR1321/MT1543*) has not been tested to see if it *tph-1* still has an increased fat phenotype. Using Oil Red staining, the new strain does appear darker just by visual inspection (figure 6 A and B). When gonadal images are analyzed with ImageJ, *tph-1* does show an increase in staining. However it is not significant within the error of the analyzed wild type images (figure 6 G). I am in the process of preparing more samples to stain with Oil Red. With more replicates and pictures to analyze, perhaps the error bars will shrink to confirm *tph-1*'s fat phenotype.

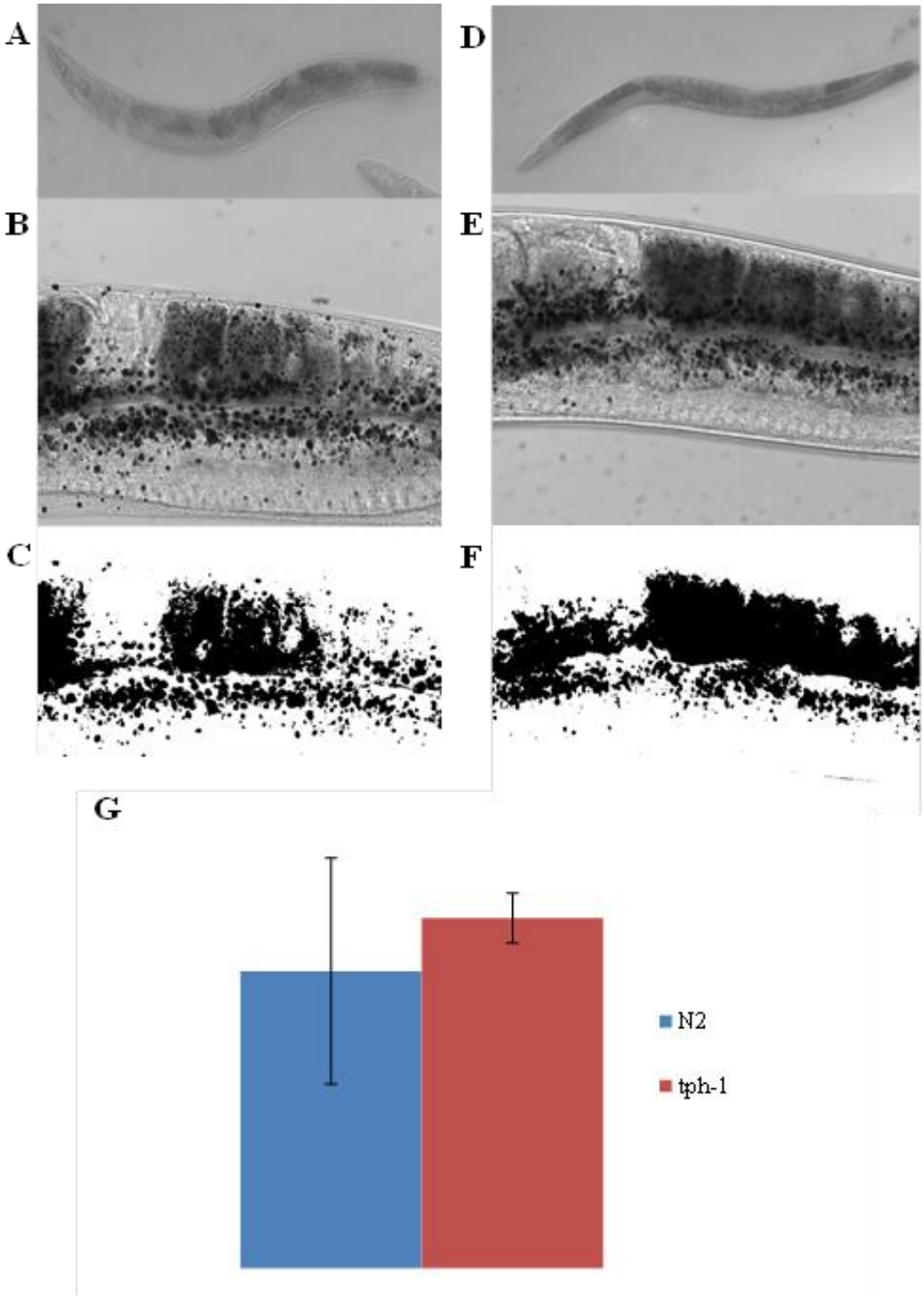

Figure 6- (A-C) Nomarski images of Oil Red Stained N2. (D-F) Nomarski images of *tph-1(GR1321/MT1543)*. Pictures were taken using GFP filter. Density Quantification was done using ImageJ. Gonad pictures (B and E) were adjusted using ImageJ so that major lipid stores are highlighted (C and F) before mean pixel density was calculated (G). 7 pictures of each strain were analyzed. Error bars represent deviation of average density calculated.

Gas Chromatography Cannot Distinguish Between Increased and Decreased Fat Storage Mutants

Once FAME parameters were established, fat mutants were tested in order to see if GC can correctly distinguish between increased and decreased fat storage mutants. Selected fat mutants have been shown previously to have increased fat content using various stains—*aak-2(gt33)* AMP activated kinase mutant, *nhr-49(nr2041)* mutation in nuclear hormone receptor controlling β-oxidation, *age-1(hx546)* phosphoinositide 3-kinase mutant, and *tph- 1(GR1321/MT1543)* outcrossed strain mutant in serotonin synthesis (Lemieux et. al 2011; Ogg & Ruvkun, 1998; Gilst et. al 2005; Ashrafi et. al 2003). The gain of function notch receptor, *glp-1(ar202)*, was previously shown to have a fat decreased phenotype while the loss of function allele, *glp-1(e2141ts)*, was shown to have a fat increased phenotype (Wang et. al 2008). The gain of function notch receptor, *glp-1(ar202)*, was previously shown to have a fat increased phenotype while the loss of function allele, *glp-1(e2141ts)*, was shown to have a fat decreased phenotype (Wang et. al 2008). Figure 7 reports the average lipid content of four experiments.

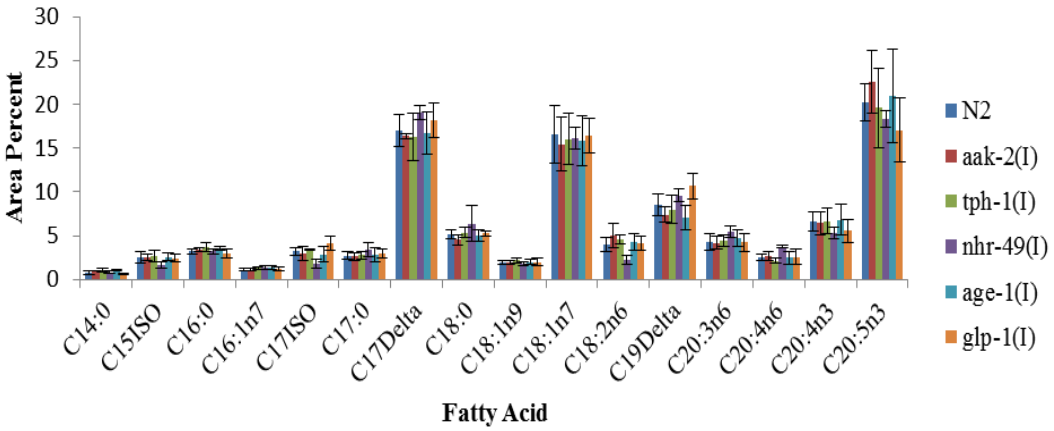

Figure 7- Average lipid content of four experiments. Each experiment contained triplicate samples of each strain. All strains were grown at 20C. (I) indicates a fat increased strain (D) fat decreased. g*lp-1(ar202)* is shown above. All FAME samples were prepared using basic method. C17Delta levels indicate all worms were fed relatively the same amount making strains comparable. Within biological error, lipid profiles are all relatively the same.

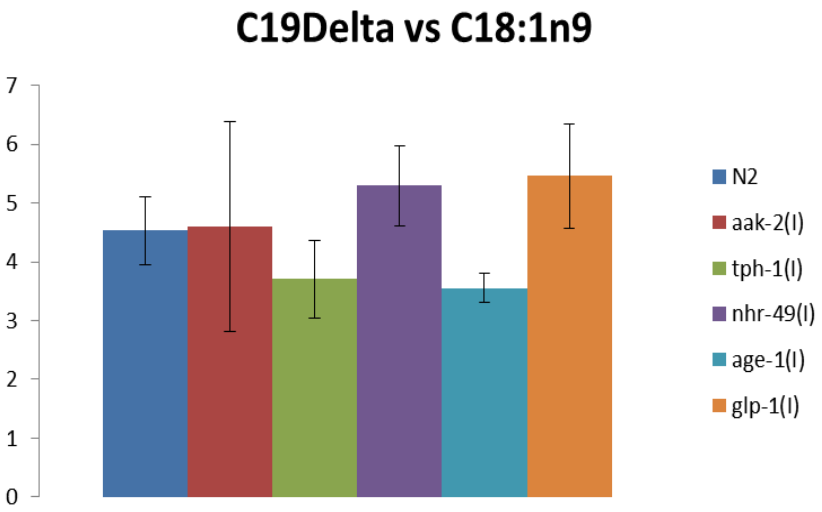

Figure 8- Average C19Δ vs. C18:1n9 ratio of four experiments (12 samples per strain total). Ratios were calculated for each experiment then averaged together. Error bars represent biological differences between experiments. All strains were grown at 20C. (I) indicates a fat increased strain and glp-1(ar202) is shown aboce. All FAME samples were prepared using basic method .This ratio was tested because these fatty acids changed the most with varying starvation duration (figure 4).

Each experiment contains triplicate samples of each strain. Looking at C17Δ, it appears that all strains were relatively fed the same since the average amount making their fat levels comparable.

Next, I compared certain fatty acids to one another in order to establish a ratio that can distinguish mutants with increased fat storage to those with decreased fat storage. Starvation data suggested the ratio between C19Δ and C18:1n9 might be able to distinguish between mutants. However, there is no

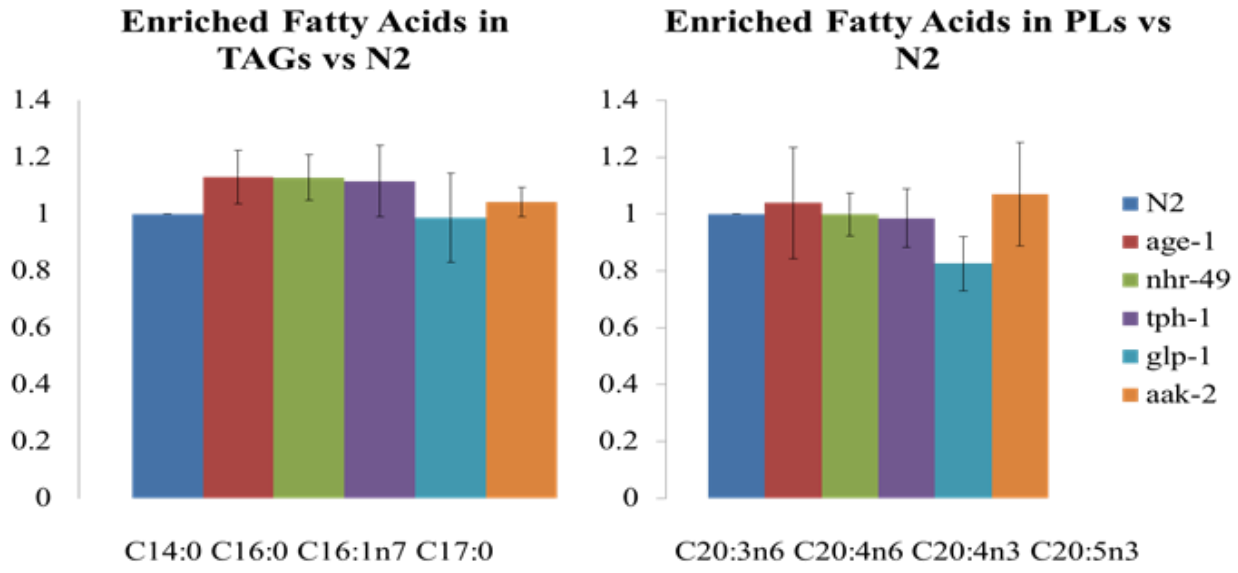

Figure 9- Fatty acids enriched in TAGs normalized to wild type amounts. The same was done for fatty acids enriched in PLs. Fatty acids that were summed together to constitute TAGs and PLs are listed below. Ratios were calculated for each experiment that averaged together. All 12 samples per stain were kept at 20C and prepared using basic method. Error bars represent biological differences between different experiments.

obvious trend using those fatty acids (figure 8). Afterwards I combined fatty acids that are enriched in triglyceride (C14:0, C16:0, C16:1n7, and C17:0) and normalized the mutant amount to the wild type amount (figure 9). The same was done with the PUFAs which are enriched in phospholipids (figure 9). Within error no significant fatty acid ratio was seen that can accurately identify fat storage mutants.

Discussion

Visualizing fat in *C. elegans* using lipophilic stains can be both subjective and variable. For example, Nile Red has been shown to poorly stain tissues known to be rich in fat. In addition, the staining of live worms can stain lysosome-related organelles producing false positives. Despite difficulties, there have been some advancements in making staining better. Previous studies showed staining fixed worms with Nile Red is better than staining live worms and the intensity of Oil Red decreases as the duration of starvation increases, making Oil Red staining a better indicator of fat (Brooks 2009; O'Rourke 2009). Using an objective technique like gas chromatography to discuss fat in *C. elegans* could eliminate the variability of staining and standardize how the fat storage mutants are described. In light of the variability of staining and the objectivity of GC, I sought to find a ratio of certain fatty acids that differs in fat increased and fat decreased mutants which can be used to qualify fat storage mutants.

First I sought out to standardize the way FAME samples are prepared. The preparation of FAMEs using potassium hydroxide (basic method) and sulfuric acid (acidic method) are both commonly used methods in the lab, but the lipid profiles generated by both methods have not been formally compared until now. Here I showed that the basic method detects more PUFAs, but the acidic method detects free fatty acids resulting in an increase of cyclopropane fatty acids in lipid profiles. Both methods have their advantages and disadvantages. Since I am interested in

being able to distinguish increased fat mutants from decreased fat mutants, it was not necessary to see free fatty acids in the worm so I chose the basic method to prepare FAMEs. Once the method was chosen, I optimized the method by setting an appropriate incubation time for the FAMEs to be generated to create the truest lipid profile of the worm.

I also found temperature does not alter lipid profiles significantly, making it easier to compare fat mutants grown at different temperatures. Experiments done at varying temperatures were done twice at two different times. Each experiment contained triplicate samples. Biological errors could be due to errors in the gas chromatograph itself. Since polyunsaturated fatty acids are long and complex, is it possible more PUFAs got stuck in the column during some GC runs which resulted in less PUFA seen in a lipid profile. One source of error may stem from the fact that the ancestry of the replicates was not tightly controlled. The first generation of the first experiment came from starved wild type worms whereas the first generation of the second experiment came from well fed wild type worms. This difference at the first generation could influence generations by epigenetic memory which may provide more or less fat in eggs. Cross generational epigenetics has already been shown to influence longevity in worms; perhaps epigenetics influences fat stores as well (Greer et. al 2011). Epigenetic regulation could influence total fat content of those worms as adults. When this experiment is repeated, the parental strain conditions will be standardized in order to make better conclusions.

Gas chromatography is sensitive enough to detect changes in lipid profiles of varying states of starvation giving me confidence that GC will detect differences in fat storage. Fat storage mutants tested were selected because they had been previously shown to have a fat phenotype using various staining methods. Because gas chromatography is capable of showing relative amounts of fatty acids present in a sample, then fatty acids enriched in triacylglycerol's

should be relatively higher in the lipid profiles of increased fat mutants. Likewise, the lipid profiles of fat decreased mutants should show decreased amount of TAG enriched fatty acids. Collecting samples and analyzing them on four different occasions, GC lipid profiles do not show any enrichment of fatty acids compared to wild type lipid profiles. In addition, I observed fat decreased GC lipid profiles for *glp-1(ar202)* which does not agree with the previously reported fat increased phenotype. All FAME samples were prepared using whole worm lysate, so it is possible that analyzing total lipid content drowns out the lipid content of TAGs. If this is true, this could explain why the enrichment in fatty acids was roughly 10% and insignificant within error. To address this, thin-layer chromatography should be performed to separate TAGs from phospholipids and then quantitate how much of each fatty acid is present in each. Doing this would confirm that the selected mutants do indeed have a fat storage phenotype. A protein control should also be added to be able to quantitate fatty acids rather than observing relative changes in fatty acid composition. A protein control will also confirm fat phenotypes in the selected mutants. Additional analysis should also be done on the phospholipid compositions. Perhaps increased fat mutants have a different phospholipid head group composition than fat decreased mutants. Then

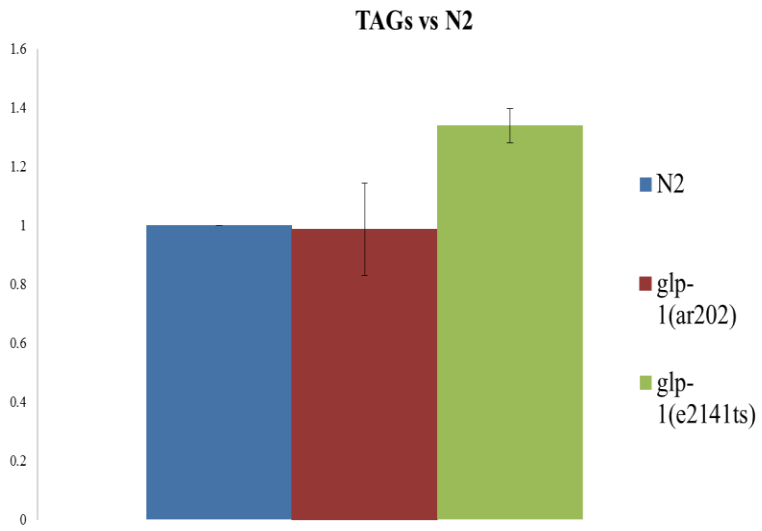

Figure 10- Preliminary analysis of glp-1(e2141ts) which is sterile at 25C. When grown at this temperature and analyzed with basic method. When TAGs are normalized to N2 content (as done in figure 9), a 30% increase is observed. Preliminary data suggests germline defective mutants have more significant changes in lipid content that can be detected using GC.

the ratio of phospholipids can be used to distinguish between fat increased and fat decreased mutants.

Age-1(hx546), aak-2(gt33), nhr-49(2041), tph-1(GR1321/MT1543); *glp-1(ar202)* are able to give rise to progeny normally. In doing so, their lipid composition may be regulated to allow fertility. Obliterating the germline may alter lipid compositions more drastically and can be seen better using GC. Early experiments to test this hypothesis use a different allele of *glp-1(e2141ts)* where there are no gonads formed at 25C. Analysis of these lipid profiles differ from the reported phenotype showing a 30% increase in fatty acids enriched in TAGs—this change is more drastic than the one previously observed (figure 10). Again, this does not agree with literature which reported *glp-1(el2141ts)* as a fat decreased allele (Wang 2008). More replicates are needed in the future to confirm this observed phenotype. In addition to germline defective mutants, dauers—larval state under harsh starvation states—should also be tested for a fat phenotype assuming this extreme state will also produce drastic GC lipid profiles. Finally, other fat mutants should be tested that are known to have a stronger fat phenotype. For instance substitution allele *age-1(mg44)* has been shown to have a stronger fat phenotype than substitution allele *age-1(hx546)* which is what I tested (Ogg et. al 1998). *daf-2* alleles *(e1370)* and *(m41)* both have substitution mutations and show drastic increases in TAGs. *eat-2*(ad465) shows a stronger fat decrease phenotype (Brooks et. al 2009; Perez & Van 2008).

Lipid profiles of fat mutants and temperature dependence experiments (done with wild type worms) did not show any variance in lipid profiles, suggesting the lipid content in worms is under tight regulation. Reasons for lipid homeostasis are unknown. One reason might be fertility; however, this is does not seem like the only possible explanation. A future project should

investigate why lipid homeostasis is important in *C. elegans* and what happens when it is disrupted. Techniques established in this study could be used to study homeostasis in the future.

Conclusion

The purpose of this study was to establish a new way to classify fat phenotypes using gas chromatography by finding fatty acids whose ratio can correctly distinguish between fat increased and fat decreased worms. In doing so, the basic method was optimized and utilized for FAME analysis using GC. I found no significant ratio exists to correctly distinguish mutants correctly; however this work does illuminate the importance of lipid homeostasis.

Acknowledgements

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